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BIOREMEDIATION: A NEW TECHNOLOGY USING INHABITANTS OF THE SOIL

An undergraduate thesis prepared
for the Honors Program by
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Abstract

Bioremediation is the use of microorganisms to clean the environment contaminated with various pollutants such as chemical wastes and petroleum-based products. The microbes use the pollutants as their carbon source and by doing so the contaminants are broken down. This paper examined the numbers of indigenous microorganisms in soil contaminated with diesel fuel and oil. Contaminant-degrading microbes were isolated and enumerated using phenanthrene in mineral media. The breakdown of phenanthrene was used as an indicator for contaminant degradation. The effects of aeration and the addition of nutrients on bioremediation processes was also studied. Soil samples used in the performed experiments were obtained from Triple M Land Farms, Inc.

Aeration and fertilization were found to quicken microbial degradation. The same results, however, were observed for controls over longer periods of time. Phenanthrene-degrading microorganisms were identified to possible genus, but were not fully identified.

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Special thanks to the Honors Program for awarding me the grant to help with research costs and to Rod McCurry for use of his laboratory equipment. I would like to thank Mr. Mark Clauson, for his advice and constant encouragement. Also, thanks to Triple M Land Farms, Inc., for their cooperation and help in attaining soil samples.

DEDICATION

This project is dedicated to my mother, Fran Fields, and to my father, Wayne Fields, for their constant support and encouragement. If not for them, I would have never had the opportunities available to me.

Introduction

Bioremediation is the use of microorganisms to clean contaminated environments of pollutants. The microbes oxidize the pollutants and use them as their carbon source. (The carbon source is the material the microorganisms use for energy.) Microorganisms usually sustain themselves on the same nutrients as humans do-- sugars, proteins, and fats. However, they are capable of using a wide variety of organic compounds. As the microorganisms use the pollutant as a carbon source, the intramolecular structure of the compound is altered, causing the pollutant to lose its toxicity and poisonous attributes that threaten the environment.

The use of microbes for bioremediation of soil contaminated with toxic pollutants has become a focus of attention because of its potential for cleaning soils at a considerably lower cost than might be achieved with other technologies, such as incineration. Bioremediation is indeed a useful tool for cleaning contaminated soil, provided that the microbes can efficiently degrade the potentially recalcitrant pollutants. Information about the chemical properties of the contaminants and the environment they are surrounded by is ever important. The process of bioremediation by natural populations of microorganisms is one of the primary methods of eliminating contaminants from the environment. A key part in the success of microorganisms for bioremediation is their adaptability. Microbes have short reproductive cycles; therefore, evolution takes place at a much more accelerated rate than for animals or plants (16). This

quick evolution, in turn, selects for advantageous traits, such as biochemical traits, giving bacteria and fungi with the ability to degrade certain substances in high concentrations a selective advantage.

Diesel fuels and oils have become an important class of environmental pollutants because of spills, improper disposal of waste, or leakage from containers. The annual global input of petroleum into the environment is between 1.7 and 8.8 million metric tons (20). A number of the polynuclear aromatic hydrocarbons (PAH), benzene-toluene-ethylbenzene-xylene (BTEX), and total petroleum hydrocarbons (TPH) are contained in diesel, gasoline, and oily by-products. Companies such as Triple M Land Farms have started to take these contaminated soils and, through correct, proper management, allowed the microorganisms indigenous to the soil to biodegrade the pollutants.

Other harmful pollutants that microorganisms have been found to degrade are xenobiotics. Xenobiotics are chemically synthesized compounds that do not exist in nature. These pose an enhanced threat because the possibility is greater that the organisms able to degrade these synthetic compounds may not exist. The most common xenobiotics are pesticides. Some of these are able to serve as carbon sources and electron donors for certain soil microorganisms. Many of these, though, are very recalcitrant and resist degradation. Much of the success with bioremediation has come in the treatment of oil, gasoline, and diesel fuel. The majority of petroleum hydrocarbons are degraded, but the heavier hydrocarbons like asphalts, waxes, and tars are very resistant to microbial attack. Researchers have even met success with the degradation of lighter halogenated aromatics and PAH's (29).

After a contaminant has been introduced into the environment, the volatile fractions of the spill evaporate quickly, leaving behind the longer chain aliphatics and aromatic compounds. These are the components that the degrading microorganisms oxidize. The microbes capable of oxidizing such contaminants do so enzymatically. Just as our bodies produce particular enzymes to breakdown certain substances, particular bacteria and fungi produce enzymes that degrade these harmful pollutants.

Bioremediation may be conducted on landfarms, in bioreactors, and *in-situ*. In landfarming, one mixes contaminants with surface soils, using a method similar to composting. Sometimes the soil is already contaminated and is simply removed and taken to a properly equipped facility to handle contaminated soil, such as a landfarm. At the landfarm, the soil is aerated, monitored, and sometimes treated with nutrients to promote growth of indigenous flora. Land treatment is the least expensive of the methods, although it requires large areas of land.

A bioreactors provides a more rapid and sometimes more efficient method of bioremediation than the other two mentioned. The bioreactor is an apparatus designed to contain a slurry (mixture) of soil, water, contaminants, and an inoculum of a microorganism. A bioreactor allows for easier control of such factors as pH, moisture, and nutrients. However, it is a more expensive method, and only limited amounts can be bioremediated at a single time.

On-site treatment, or *in-situ* treatment, has the advantage of no excavation or removal of what is to be bioremediated. The growth of indigenous populations is used to degrade the contaminants, and sometimes even promoted with the addition of nutrients, as in the case of

the oil spill in Prince William Sound. Controlling abiotic factors, of course, is much more difficult with *in-situ* and landfarming techniques (29).

Factors such as oxygen availability, nutrient concentrations, moisture, pH, and addition of nutrients all affect the biodegradation process (20). For many years municipal waste-water facilities have used microorganisms to help oxidize organic pollutants in the water. It is evident that microorganisms aid in removing unwanted pollutants from the environment. Many of these contaminants are caused by man, and if not for these helpful bacteria and fungi, man would be sinking in his own pollutants.

Value of Bioremediation

Industry and the public have become more interested in new technologies in the field of treating/cleaning hazardous waste materials. Pressure from politicians and the general public increases as society demands the clean-up of contaminated environments. The Environmental Protection Agency (EPA) is passing new bans regulating the disposal of hazardous waste at landfills, and public outcries are raising opposition to the process of incineration (16). Because of these pressures, along with the high cost of conventional methods of waste disposal, bioremediation is seen as an attractive technology. Although bioremediation was first used fifteen years ago to clean hydrocarbon-contaminated aquifers, it has recently gained popularity (16).

One of the important values of bioremediation is that it is a natural process, using indigenous microflora in the soil to rid the environment of unwanted pollutants. The microorganisms completely destroy the

contaminant, leaving behind carbon dioxide and water, without destroying the surrounding environment. Also, it is very easy to implement bioremediation on site, as with large-scale oil spills. Plus, bioremediation solves the problem of contaminated soil, whereas conventional methods merely relocate or postpone it.

Bioremediation also helps solve the landfill crisis. Many of the plastics, polyurethane, polystyrene, and other polymeric substances are rapidly filling up all available landfill space (6). Much research is being spent to develop building materials that are more biodegradable. Landfill space is also going to soil that is contaminated with toxic pollutants. Instead of paying the high prices of chemically cleaning the soil, companies are taking the cheaper alternative of dumping the toxic soil and covering it up. However, because of new regulations, companies are having to find ways to clean the soil and make it safe for the environment. Bioremediation is the surest, safest, and cheapest method to clean contaminated environments.

An article which compared the costs of bioremediation and conventional methods found that bioremediation costs somewhere between \$50-\$100 a ton, chemical stabilization costs \$50-\$100 a ton, on-site incineration costs \$300-\$400 a ton, and off-site incineration can cost up to \$1000 dollars a ton (19). In 1991 the bioremediation market was about \$60 million dollars. Based on annual growth rate, the market is projected to grow to about \$150 million by 1995 (29). Not only is bioremediation the least expensive mode of cleaning up contaminants, it is the only one that actually rids the environment of the pollutants.

Along with universities and research laboratories, there are new companies forming all over the country that are using bioremediation

techniques to help clean up wastes. Environmental consultant William T. Lorenz states that companies are getting contracts of more than a million dollars for bioremediation (19). New companies such as Bioremediation, Inc. (San Diego), Groundwater Technology, Inc. (Norwood, MA), Cytoculture International, Inc. (Point Richmond, CA), Envirogen, Inc. (Lawrenceville, NJ), Triple M Land Farms, Inc. (Franklin, KY), and older companies like IT Corp., General Electric Co., and the Exxon Oil company are using bioremediation.

There are some limitations to bioremediation. If nutrient additions are made to aid the growth microbes, one must keep in mind the effect of amendments on the surrounding environment. Bioremediation must be monitored correctly to ensure that metabolic pathways do not produce toxic intermediates. Also, bioremediation seldom removes all of the target waste, although the EPA has set standards describing the acceptable and non-acceptable levels of major contaminants in the environment.

Another problem with the field of bioremediation is its lack of a standard strategic approach on proper research methods to use in the environment (16). A recent meeting of the American Academy of Microbiology concluded that the field holds great optimism but needs more of a coordinated interdisciplinary research attack. However, with its growing popularity and research availability, bioremediation promises to be an exciting and certainly helpful field. The real test is implementing laboratory results out in the field, to take advantage of the enzymatic pathways of the microorganisms.

Bioremediation can be very difficult to prove because of all the different biotic and abiotic parameters in different environments. There is a delicate and intricate relationship between nutritional requirements,

biological processes, and the physical conditions of the environment.

When a sample is removed and transported to a lab, the conditions cannot be assumed to be unaltered from the same soil out in the field (22). For the potential of biodegradation to be proven, microbial metabolism of the contaminant must have occurred, environmental parameters considered, and responsible microorganism(s) isolated (22). Also, diminished concentrations of contaminants in the field, increased numbers of contaminant-degrading microorganisms, and the presence of intermediate metabolites and reactants attest to bioremediation. To be absolutely sure that *in-situ* bioremediation is taking place, a researcher must perform experiments that simulate the actual outdoor environment.

Microbial Breakdown of Contaminants

One reason for the varying degrees of microbial degradation is due to the different susceptibility rates of different compounds. Hydrocarbons usually rank in the following order of decreasing microbial susceptibility: n-alkanes > branched-alkanes > low molecular weight aromatics > cyclic alkanes (21). Light aromatics usually degrade faster than high molecular weight aromatics and polar compounds. These standards are not universal, and many variations have been observed. A large factor affecting susceptibility is the constituent groups attached to the compound. Also, the ability of the microorganism to produce emulsifiers is important. By producing emulsifiers and biosurfactants, bacteria greatly increase their ability to uptake and degrade the present contaminants (21). A study found that the use of biosurfactants helped

Pseudomonas spp. to degrade contaminants with limited water solubility (32).

The following categories are the main constituents of hydrocarbons: paraffins, cycloparaffins, aromatics, and naphtheno-aromatics. The paraffins, which are straight chain and branched alkanes, usually comprise 25% of crude petroleum. These can include simple alkanes such as methane or complex species such as n-tritetracontane. The cycloparaffins are cycloalkanes and represent 30-60% of crude petroleum. Most of the cycloalkanes are monocyclic, but compounds containing up to ten rings have been isolated. Aromatic hydrocarbons are much less prevalent in most petroleums. They are cyclic structures with different properties caused by delocalized electrons in their benzene rings. The naphtheno-aromatics are cycloalkanoaromatics and usually have structures resulting from a fusing of the other three classes. The naphtheno-aromatics are found in the high boiling fraction of petroleum (7). Along with these main constituents, petroleum also contains components such as naphthenic acids, phenols, thiols, heterocyclic nitrogen, sulfur compounds, and metalloporphyrins (2).

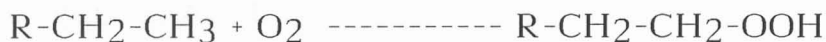
The biodegradation of oils and fuels varies with the constituents of the pollutant. The type and size of the hydrocarbon molecules decide readily its susceptibility to biodegradation (2). The longer the alkane chain, the less degradable the molecule becomes; the same holds true for the branching of the molecule. Alkane chains can become too large to serve as a carbon source for microorganisms. Aromatic compounds are degraded more slowly as are alicyclic compounds. However, microorganisms are capable of degrading such recalcitrant compounds via

co-metabolism, when two microbes possess complementary metabolic pathways for a specific compound (2).

As stated by Atlas and Bartha (2), the initial attack for aerobic microbes occurs with the use of monooxygenases or dioxygenases. In one instance, oxygen is incorporated into the alkane, producing a primary alcohol, water, and a reduced form of nicotinamide dinucleotide phosphate (NADP, which serves as an electron donor).



In a second case, both atoms of oxygen are incorporated into the alkane, producing a hydroperoxide that is then transferred to an alcohol and water.

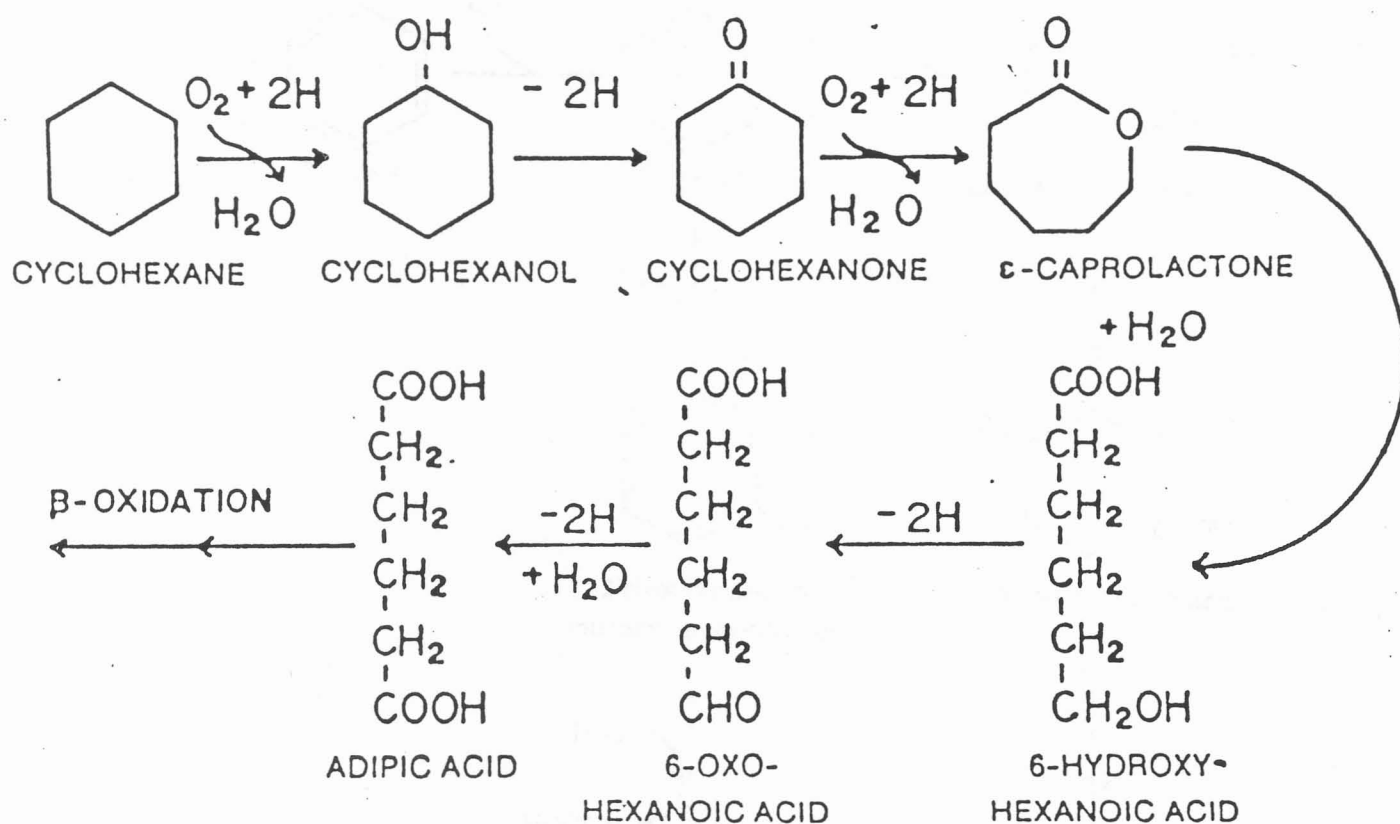


Usually the alcohol is further degraded to an aldehyde and fatty acid (the fatty acid being a source of energy for the microbe by way of the *B*-oxidation sequence). In this sequence, the fatty acid is converted to its acetyl-CO-enzyme A form. These co-enzyme A units are converted to carbon dioxide through the tricarboxylic acid cycle. In anaerobic degradation, it is suggested that dehydration occurs, leading to the formation of an alcohol, then an aldehyde, and eventually a fatty acid. The process of aerobic degradation is much more documented and more easily studied (2).

In the case of no terminal methyl groups existing, oxygen is inserted and a secondary or tertiary alcohol is formed. Dehydrogenation leads to a

ketone, and further oxidation forms a lactone. The lactone is hydrolyzed, and the hydroxyl group is oxidized to an aldehyde and carboxyl group. These intermediates are then metabolized by the *B*-oxidation sequence of the bacteria (see Fig. 1) (2).

Figure 1. Cyclohexane is used as an example for the degradation process of an alicyclic hydrocarbon (2).



Aromatic hydrocarbons are extremely recalcitrant, and microbes have difficulty in degrading them. If degradation is possible, the hydrocarbon is hydroxylated to form catechol, and the aromatic ring is opened by ortho cleavage. Then the molecule undergoes further breakdown to common tricarboxylic cycle intermediates, succinic acid, and acetyl CO-enzyme A (see Fig. 2 and Fig. 3). Another route may be taken in which metabolism leads to formic acid, pyruvic acid, and acetaldehyde (2).

Figure 2. Microbial degradation of a benzene ring, showing two different pathways used by microbes (2).

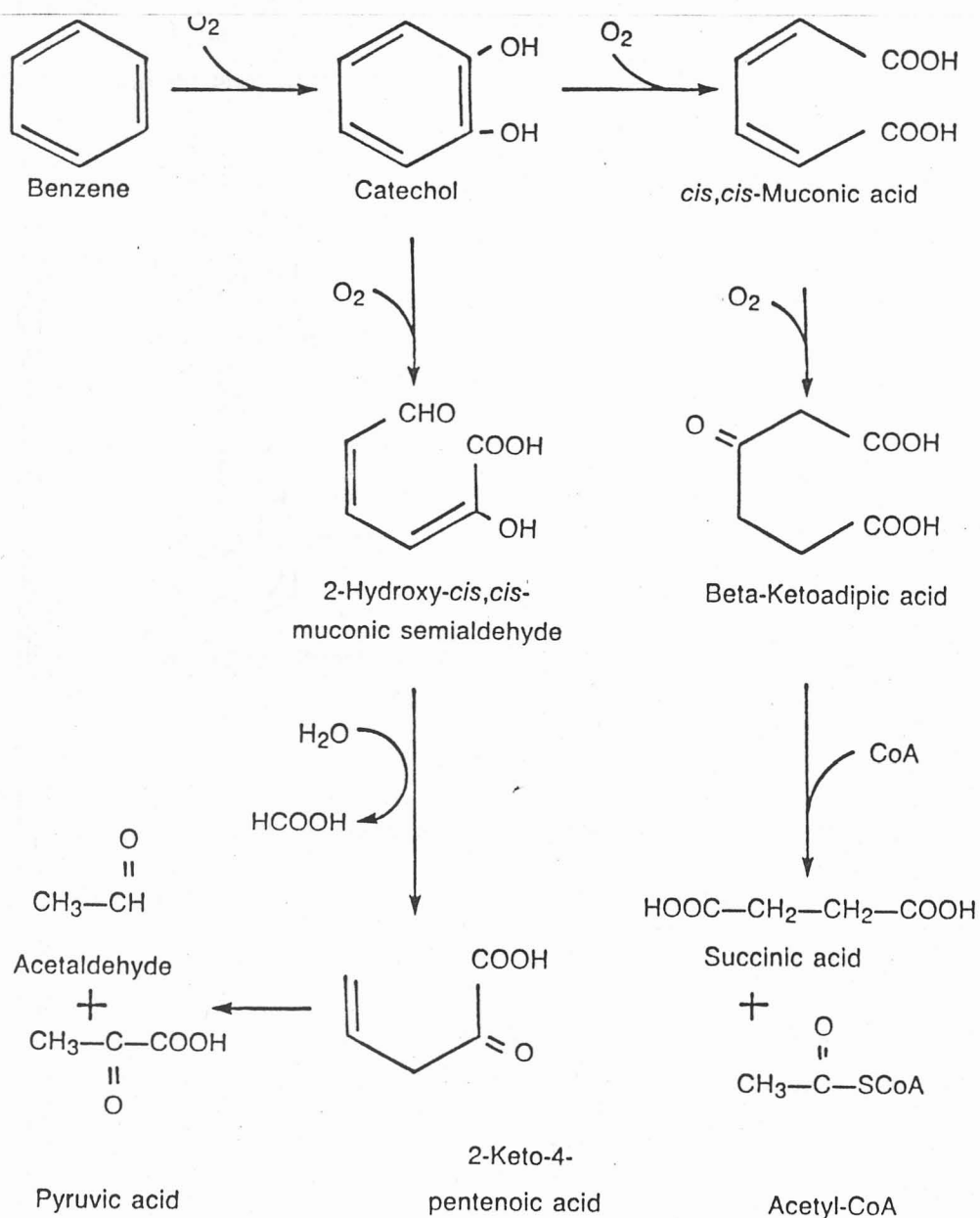
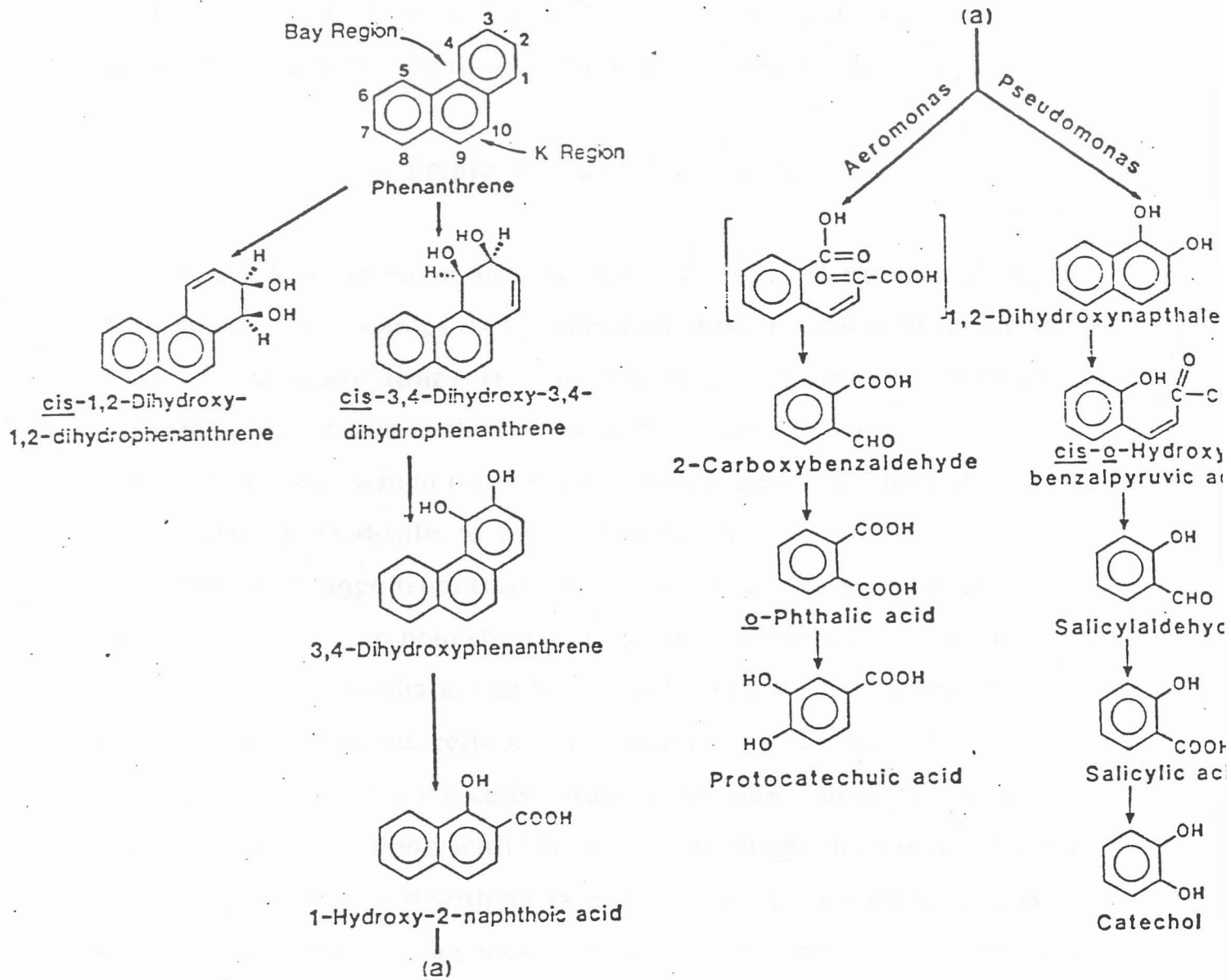


Figure 3. Two different pathways for the oxidation by bacteria for the compound phenanthrene (9).



These processes are obviously advantageous and possess great potential. However, the abiotic factors, such as pH, moisture content, soil type and weather, affecting microbial bioremediation are in nature's control and serve as limiting factors. But operations such as landfarms and bioreactors serve as an answer to one of the environment's pressing

problems. With the use of landfarms and bioreactors, along with continued research, at least some of the factors affecting bioremediation can be controlled. There are a plethora of different factors that play a role in the ability of the microbes to degrade the contaminants.

Triple M Land Farms, Inc.

Triple M Land Farm, Inc., located in Franklin, Kentucky, is one of the pioneering companies using bioremediation to treat contaminated soils. The company utilizes the capabilities of indigenous soil microbes to degrade contaminants in the soil. The farm receives a load of contaminated soil, which is placed into a prepared cell where it is spread out and tilled periodically to allow for aeration.

The cells range from three to four acres in size (see Fig. 4). A storage cell is used to hold shipments of soil that are waiting to be placed in one of the bioremediation cells used for actual decontamination. The smaller areas below the cells are retention ponds. These collect any water that drains from the cells. Water collection is achieved by the sloping of the cells, along with sub-surface drainage lines beneath them.

Every cell is lined with an average of twenty-three feet of clay, which is very resistant to penetration and serves ideally as a protective lining. This natural clay lining serves as the unsaturated zone. Each cell is also surrounded by berms, that is, elevated soil enclosing the cell. Each cell has a water collection tank (sump), a lysimeter, a treatment zone core, a monitor well, a sub-surface drainage system, a treatment zone, and an unsaturated zone (see Fig. 5). The treatment zone is the area of the cell where contaminated soil is spread.

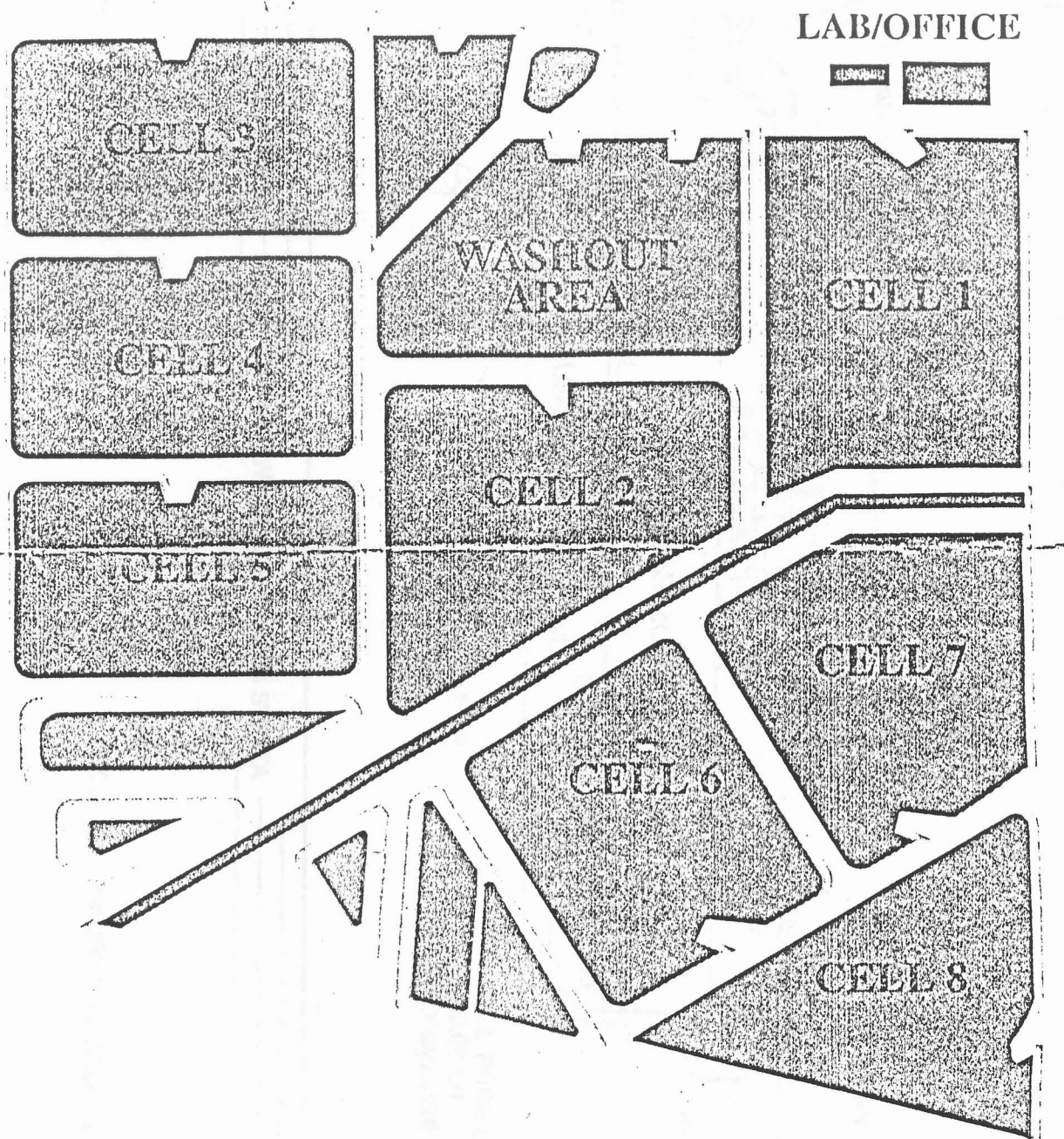


Figure 4. Design and layout of Triple M Land Farms, Inc.

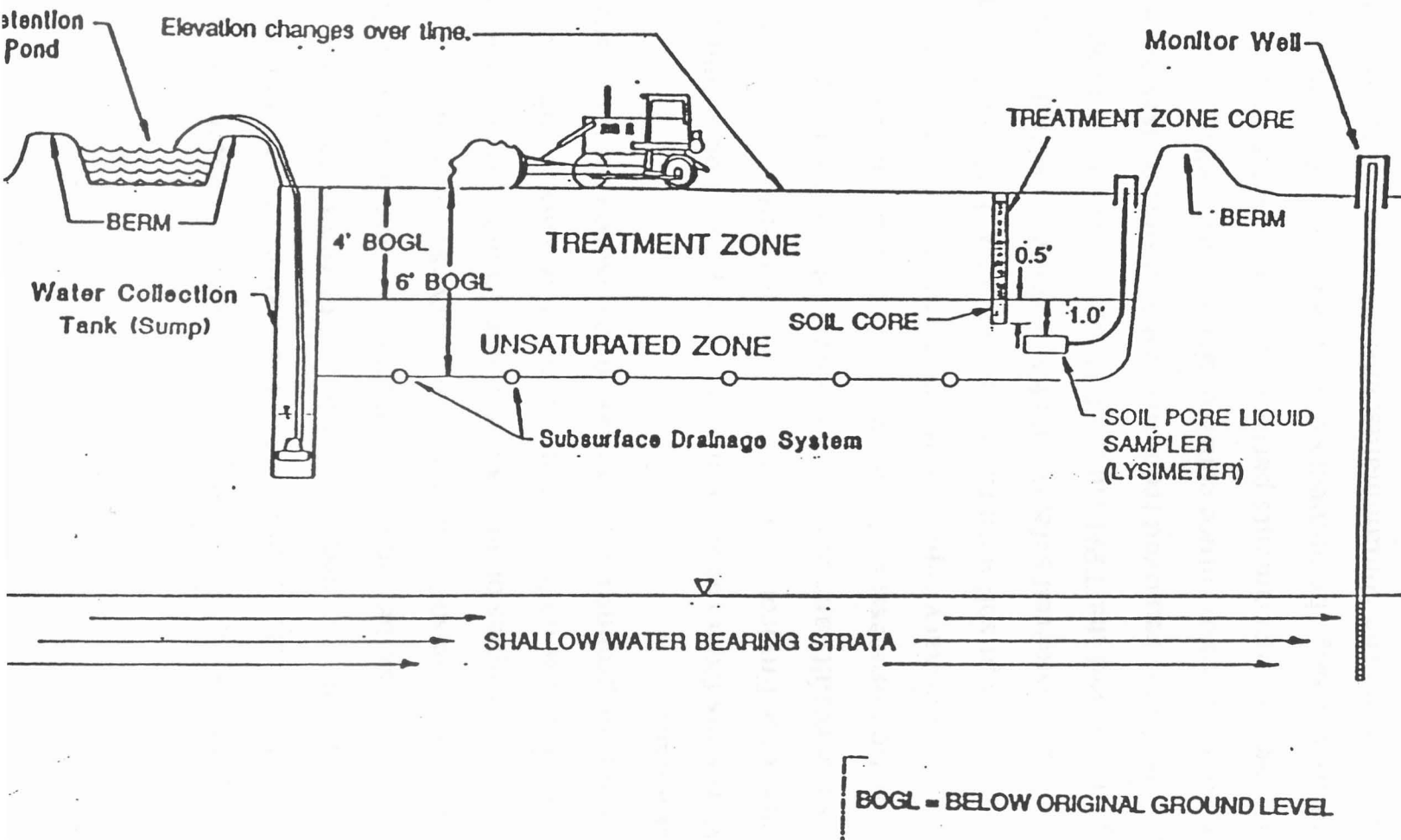


Figure 5. Design of an individual constructed cell at Triple M Land Farms, Inc.

The subsurface drainage system collects all water that falls on the soil above. This water is pumped to the water collection tank and then to the retention pond for easy monitoring. The treatment zone core is where a sample of soil can be removed and analyzed periodically for levels of contaminants in the soil. The lysimeter serves as a permanent sampling station where samples of soil can be taken from the unsaturated zone to ensure that there is no leaking of the contaminants. From the monitor well samples of groundwater are taken to ensure that there are no traces of contaminants.

All results from the sampling of the soil must be submitted to the Department for Environmental Protection of Kentucky. The department monitors the results and ensures the safety of the company's bioremediation processes. Triple M, prior to acceptance of any soil, receives a preliminary report of an analyzed sample. The company is permitted to accept soil with readings for BTEX, PAH, and TPH. For a sample to be considered safe, the BTEX reading must be 1 ppm, the PAH reading 1 ppm, and the TPH value of 10 ppm. When the proper level is attained, the soil is removed from the cell and stored elsewhere on the property to be used in future construction of new cells.

During the experiments performed in this thesis, information was obtained concerning the numbers of microorganisms in the soil, their ability to degrade the contaminants, and different parameters that affect their growth. Triple M possibly can use this information for faster and more efficient bioremediation.

Abiotic Factors

The concentration of the contaminants in the soil limits the ability of the microflora able to degrade it. In general, if the concentration of the contaminant is between 0.5-5%, degradation is observed. Higher concentrations tend to be overly toxic to the microorganisms. As stated earlier, the abiotic parameters of the soil influence the condition of the microorganism as well as the soil itself.

Temperature influences both the contaminant and the microorganism. Temperature affects the nature and activity of many contaminants while also directly affecting the microbe's metabolic pathways. Leaky and Colwell (21) stated that degradation declines with decreasing temperature. The physical characteristics of many contaminants change with temperature, and a lower temperature decreases the metabolic pathways of most microorganisms (most being mesophilic). The maximum temperature thought to be advantageous for growth of microbes is between 30-40° C. Temperatures above this tend to cause many contaminants to become increasingly toxic (4). Thus, climate and season are important factors in determining the rate of bioremediation.

Oxygen, obviously, is crucial to aerobic microbial degradation. The initial step in degradation of hydrocarbons by most microbes involves the oxidation of the contaminant by the enzyme oxygenase. The condition of the soil, such as microbial oxygen consumption, soil type, and moisture content can influence whether degradation progresses (21). There have been many studies showing that anaerobic degradation of hydrocarbons is important. One study proved anaerobic degradation by denitrifying

bacteria (24). Other microorganisms have recently been found to mineralize benzene under anaerobic conditions (15). Anaerobic degradation is vastly important, especially at underground sites. However, it is by the process of aerobic degradation that bioremediation occurs once the soil is at the land farm. By tilling the soil, one maintains the concentration of oxygen throughout the soil, thus allowing for optimal aerobic conditions for the microorganisms. Along with aeration, the addition of nitrogen and phosphorous have been found to accelerate bioremediation. A study conducted on waste-water ponds showed significant increases in bioremediation when aeration and fertilizers were used (11).

Water concentration of the soil also limits bioremediation. Optimal water saturation has been found to exist between 30-90%(21). Certain contaminants affect the water-holding capabilities of soil and play a role in limiting the amount of necessary water available to the microorganisms.

Soil pH, unlike aquatic ecosystems, can be highly variable. Most organisms prefer a pH around neutrality, with some yeasts and molds being more tolerant of acid conditions. Therefore, extremes in pH would have negative effects on the microorganisms.

A variety of bacteria has been isolated as degrading a plethora of contaminants produced by man, from hydrocarbons to chlorinated biphenyls. *Achrombacter*, *Acinetobacter*, *Alcalegines*, *Arthrobacter*, *Bacillus*, *Flavobacterium*, *Norcardia*, and *Pseudomonas* spp. are tending to be the most dominant contaminant-degrading bacteria. The degree to which these and other microorganisms degrade hazardous contaminants

in the soil and water has been the target of much research in the last few years.

In a study performed by Dibble and Bartha (13), the parameters of soil moisture, pH, mineral nutrients, and incubation variables were found to be important factors affecting the process of bioremediation. Other researchers proved the ability of certain bacteria (*Arthobacter* and *Pseudomonas* spp.) to degrade oily waste in soil. The bacteria numbers depended upon soil pH, moisture content, temperature, and the concentration of the oily waste (20).

Bioremediation in the Future

Recent studies have focused on genetic mechanisms responsible for microbial degradation. The adaptation of microorganisms combined with their ability to pass along genetic information to other microbes enables them to degrade a variety of carbon sources. Through the ability of adaptation, genes responsible for degradation pathways are induced by environments with concentrations of a particular contaminant. Via gene transfer and mutation, this genetic information can be passed from generation to generation of microorganisms. Researchers have produced a recombinant strain of *Escherichia coli* containing genes from *Pseudomonas oleovorans* that encoded for the ability to degrade p-xylene (12).

By using information obtained from the above techniques, one can introduce genetically altered or engineered microorganisms into a contaminated environment, the organisms being engineered to degrade a certain contaminant. *Pseudomonas* spp. have been engineered with

recombinant DNA plasmids to degrade 3-chlorobenzoate, 4-methylbenzoate, toluene, and 4-ethylbenzoate (25).

The advantages of using genetically engineered microorganisms are endless, and the prospects for ridding the environment of xenobiotics are certainly enhanced. Nevertheless, great care must be taken when engineering new microorganisms or altering existing ones. The possibility for mutants and microorganisms developing harmful effects on the environment must be monitored carefully.

A multitude of techniques has been used successfully in identifying genes encoding for hydrocarbon-catabolic pathways. DNA probes are a main procedure developed for identifying a certain sequence of a microorganism's genes. DNA probes identify specific sequences in a microorganism's genome responsible for degradation abilities. A microorganism's DNA is digested with enzymes (endonucleases), separated, and combined with the probes. If hybridization occurs, a match is made, and one knows the genome contains genes responsible for hydrocarbon degradation. Such techniques as colony hybridization, dot blot hybridization, and polymerase chain reaction identify genes and plasmids that enable the microorganism to biodegrade. The polymerase chain reaction can be used to find and differentiate rRNA genes in mixed cultures (27). Working with a mixed culture is less laborious than having to isolate the microorganisms. The polymerase chain reaction can be used to amplify DNA from microbial populations in soil (8). These methods are also used in monitoring the distribution and uptake of an inoculated plasmid into the soil (26). These techniques allow for measurement of the genes and plasmids that enable microorganisms to degrade hydrocarbon contaminants.

Plasmids are highly mobile forms of DNA which can be transferred between bacteria via conjugation or transformation (6). The plasmid usually encodes for beneficial genetic information. In this case, the genetic information enables the bacteria to degrade the contaminants in their surrounding environment. By using plasmids, bacteria and sometimes eukaryotic microbes pass DNA among themselves(20,39). Plasmids of this type are expressed when the microorganism comes into contact with a particular substrate, thus increasing its overall frequency in the microbial population. Further studies are needed to confirm the participation of plasmid DNA in the ability of microbial populations to degrade present hydrocarbon contaminants in the environment.

Overall, bioremediation is proving to be useful in removing contaminants from the environment. Research in this area must be continued to ensure better bioremediation procedures for the future. Microorganisms have existed on earth for billions of years, constantly evolving and adapting. Their success in survival is proving to be an answer to ours.

Research studies for the most part seem to agree on the factors affecting bioremediation and the difficulty of trying to prove that bioremediation is responsible for the exhibited removal of contaminants. The combination of biotic and abiotic parameters that affect bioremediation are numerous, and trying to duplicate these parameters in the laboratory is difficult. This report shows that a decrease in the levels of contaminants at Triple M Land Farms, Inc., was partly due to populations of indigenous flora of microorganisms found in the soil. The microbes were enumerated, isolated, and classified as to whether they were petroleum biodegraders. Biodegradation was also monitored and

correlated with abiotic parameters, such as fertilization and aeration of the contaminated soil.

Materials and Methods

Sample collection and processing. Contaminated soil samples were obtained from Triple M Land Farms, Inc., in Franklin, Ky. The first collection, 11-18-92, came from an underground storage tank containing diesel fuel. The second sample was obtained on 1-19-93 and consisted of soil from an oil spill in Arkansas. Both soil samples were tested by independent laboratories to determine initial hydrocarbon concentration.

Soil samples were collected in polyethylene bags from depths between 0 to 10 cm, packed on ice for transport to the laboratory, and stored at 4 C until processed(30). Soil samples were prepared for dilutions within 12 h upon collection. Serial dilutions were prepared from homogenations by adding 11 g (dry weight) of sieved soil(2 mm mesh size) to 99 ml of 0.85% NaCl and mixing it three times in a Waring blender at low speed for one minute each with the addition of 0.05% Tween 80(14,30).

Bacterial counts. The enumeration of total bacterial counts was determined by the most probable number (MPN) technique. Serial dilutions were inoculated into peptone-yeast extract broth and incubated at 26 C for four weeks (14). Serial dilutions were also spread-plated on peptone-yeast extract with addition of 2% agar in triplicate to determine a total plate count. Yeast and molds were enumerated along with actinomycetes, utilizing Malt Extract Agar (Difco) and Actinomycete Isolation Agar (Difco) with the addition of glycerine, respectively.

Prepared plates were inoculated in triplicate and incubated for eight days at 26 C.

A MPN determination was also performed to enumerate bacteria able to degrade phenanthrene by adding phenanthrene into the medium (23). Phenanthrene was dissolved in ethanol and added to 5 ml of mineral medium to obtain a final concentration of 0.5%, then autoclaved at 15 psi, for 15 minutes at 121 C. The most-probable number determinations were carried out as described by Alexander (1).

Phenanthrene plates were used to isolate and enumerate the possible hydrocarbon-degrading microorganisms in the soil. From samples collected on 1-19-93, phenanthrene overlayer plates (3) were prepared and incubated at 26 C for twenty-six days. The plates were observed for phenanthrene degradation weekly.

Isolates from phenanthrene plates: Colonies exhibiting a halo because of the clearing of the phenanthrene were picked and streaked for purity on Tryptic Soy Agar (Difco) and incubated at 26 C for 24 to 48 h. Pure colonies were characterized into groups according to colony characteristics, stain reactions, and biochemical tests.

Addition of Fertilizer. Five experimental freezer bags (Reynold's 10 9/16 X 11 in.) were prepared by placing 1 Kg (wet weight) of soil into each bag (5,18). Bag one was a control and had no additions, 2% HgCl₂ was added to bag two (31), bag three had 2% fertilizer (10-10-10) added, bag four was aerated for the four-week process, and bag five had a combination of 2% fertilizer and aeration. Aeration was conducted by shaking the contents of the bag once every two days. All experimental freezer bags were maintained at 26 C. This protocol is shown in table 1.

Bag #	Treatments
Bag 1	Control
Bag 2	2% HgCl ₂
Bag 3	Aeration
Bag 4	2% Fertilizer
Bag 5	Aeration/2%Fertilizer

Table 1. Treatment of each freezer bag containing 1 Kg of soil.

On day 13 and 30 soxhelt extractions were prepared from each of the five soil samples in the freezer bags. Extractions were carried out according to method 9071 as used by the state of Kentucky. An extraction of the oil and grease portion was carried out with silica gel to achieve solely a hydrocarbon portion (28).

Isolation of Mixed Colonies of Biodegraders. Isolation of phenanthrene degrading bacteria was achieved by using phenanthrene in a liquid mineral medium (23) in 250 ml erlenmeyer flasks. The flasks received a 5g sample of soil for inoculum and were incubated at 26 C on a rotatory shaker (~200 rpm) for seven days (17). Microorganisms that grew in the mineral media were streaked for purity on tryptic soy agar and incubated at 26 C for 24 to 48 h.

RESULTS

Bacterial Counts: The first soil sample taken had a total BTEX concentration of 6.9 ppm. Total actinomycete count was 1.9×10^4 CFU/g. Different colony types were observed demonstrating pigments, also some exhibiting browning of the medium because of tyrosinase production. Molds and yeasts were found to be 6.3×10^3 CFU/g, the

majority of which were molds. Heterotrophic bacteria counts were found to be between 7.1×10^4 CFU/g with high occurrence of *Bacillus cereus* var. *mycoides*. These were relatively low counts and were probably due to the presence of hydrocarbons. No phenanthrene degraders were observed on the overlayer plates.

A MPN was employed to enumerate both simple heterotrophs and phenanthrene degraders. Simple heterotrophs counts were 1.2×10^5 CFU/g. A black discoloration observed in the first two dilutions of the MPN medium was possibly due to sulfate reduction. Phenanthrene degraders were found to be 1.5×10^4 CFU/g, according to the MPN technique, after four weeks of incubation at 26 C. These protocol are shown in tables 2 and 3.

PLATE COUNTS	Sample 1- CFU/g	Sample 2-CFU/g
Actinomycete	19000	13000
Molds/Yeasts	6300	230
Total Aerobic	71000	15000

Table 2. Numbers observed from plate counts for each soil sample.

MPN	Sample 1-CFU/g	Sample 2-CFU/g
Simple Heterotrophs	12000	12000
Phenanthrene Degradars	15000	880

Table 3. Numbers observed from MPN technique for each soil sample.

In the second soil sample, actinomycete counts were 1.3×10^4 CFU/g, the majority being pink, white, and crusty white colony types. Molds and yeasts were 2.3×10^2 CFU/g. The simple heterotrophic count was determined to be 1.5×10^4 CFU/g. MPN's were observed after four weeks and gave a count of 8.8×10^2 CFU/g for microorganisms able to degrade phenanthrene. When compared to controls, the phenanthrene in the positive tubes appeared to have a different appearance. The heterotrophic count using the MPN was 1.2×10^4 CFU/g, with the first two dilutions exhibiting a black discoloration as noted in the first soil samples.

Phenanthrene overlayer plates were observed weekly up to 26 days, and phenanthrene degraders were found to be 1.7×10^3 CFU/g. From these overlayer plates, nineteen colonies were picked and streaked for purity. Eight isolates were obtained that clearly exhibited a definite halo on the phenanthrene plates, denoting phenanthrene degradation.

Isolates from Phenanthrene Plates: On the basis of colony characteristics and biochemical properties, bacterial isolates were classified into groups, respectively. Biochemical data were obtained from API 20E strips, but failed to identify any of the soil isolates. Isolates were run through a series of tests to determine genus (10) (table 4). Isolates 3 and 17 appeared to belong to the actinomycete group. Both displayed hard, leathery colonies. Isolate 3 had a regular, uniform colony type while isolate 17 had an irregular type. Isolate 3 also produced a brown discoloring of tryptic soy agar. Since neither produced aerial hyphae, they were placed in the genus *Micromonospora*. Isolate 7 was motile, an off-white color, and oxidase positive. Being fermentative, it was designated as an *Aeromonas* spp. Isolates 1,2,5,9, and 14 were all yellow,

Isolate #	Gram rxn.	Oxidase	Catalase	Starch	Nitrate	Motility	Endospore	O/F	Colony Type	SO ₄ Reduct.
1	G-	-	+	-	-	+	+	O/F	yellow	-
2	G-	-	+	-	-	-	+	F	yellow	-
3	-	-	+	-	+	-	-	O/F	hard, white	-
5	G-	-	+	-	-	+	-	F		-
7	G-	+	+	-	-	+	-	F	cream	-
9	G-	-	+	-	-	+	-	F	yellow	-
14	G-	-	+	-	-	+	+	F	yellow	-
17	-	-	+	+	+	-	-	O/F	hard	+

Table 4. Characteristics and results of biochemical tests performed on isolates.

some producing brown pigmentation on tryptic soy agar. These isolates were either Coryneform bacteria or perhaps *Xanthomonas*. Further tests are being performed to identify these microorganisms.

Addition of Fertilizer: The concentration of hydrocarbons in the soil showed a decline at both the 13 d and 30 d periods. Initially, bags 1,3, and 4 contained the highest hydrocarbon concentration, with bag 5 exhibiting the greatest decline in hydrocarbon concentration (Fig. 6). All bags showed a continued reduction in hydrocarbon concentration after the 30 d period. Bags 1, 2, 3, and 4 showed more extensive declines after 30 d, while the contaminants in bag 5 were reduced more slowly.

Isolation of Mixed Colonies of Biodegraders: Microorganisms isolated from the mineral medium with phenanthrene were purified on tryptic soy agar. These isolates were stored on slants for later experimentation on phenanthrene-degrading ability and identification.

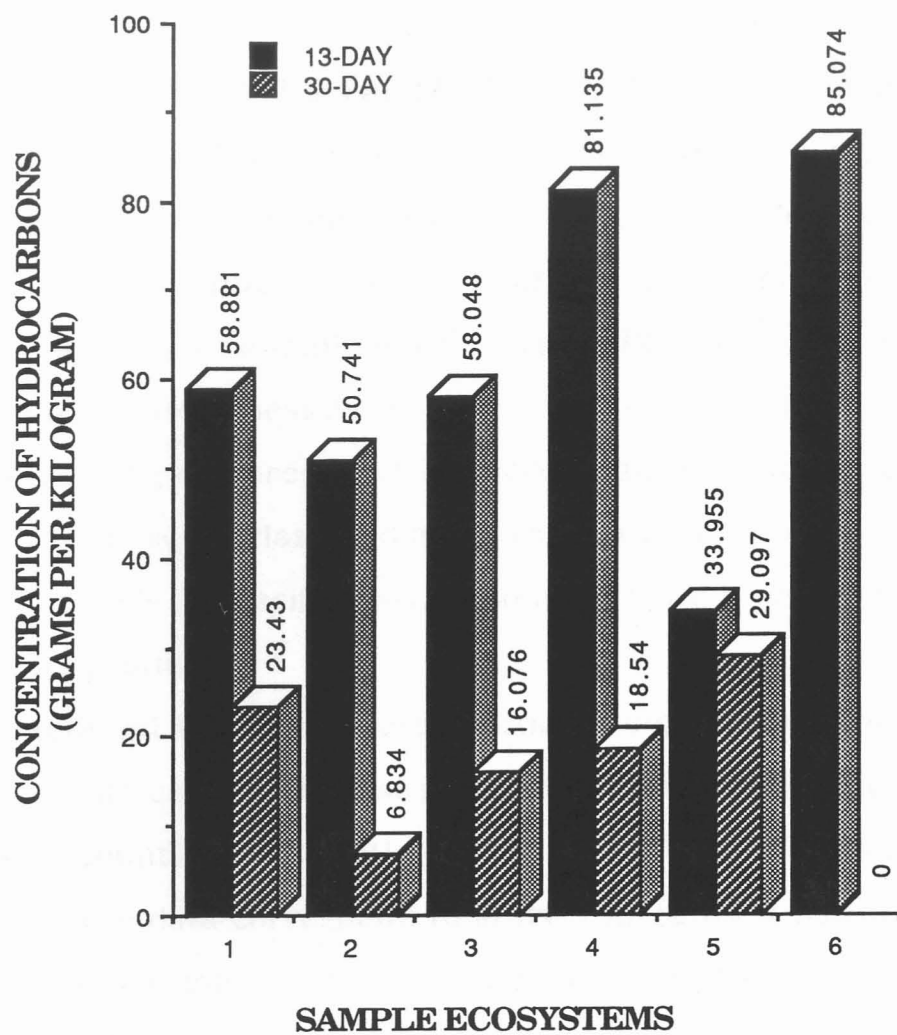


Figure 6. Hydrocarbon concentration of freezer bags after 13d and 30 d soxhelt extractions.

DISCUSSION

In this study, bacterial counts were performed on soil samples contaminated with BTEX's and PAH's. In the performed enumerations, actinomycetes, molds/yeasts, simple heterotrophs, and phenanthrene-degrading microorganisms were selected for. The degraders were selected for using phenanthrene, a polynuclear aromatic hydrocarbon (PAH) consisting of a triple benzene ring. Phenanthrene was utilized because of its usefulness as an indicator and its inability to volatilize at incubation temperatures (26 C). Microorganisms capable of degrading phenanthrene were classified as petroleum decomposers because of phenanthrene's similarities to compounds found in diesel fuels and petroleum products.

Bacterial Counts: Bacterial counts were determined using both the total plate count and most probable number techniques. It was found that MPN counts gave slightly higher counts than those obtained on plates. These data correspond to Mills et al. (23). It has also been shown(10), that lower counts are achieved when high-nutrient media is used. To achieve numbers that represent the actual indigenous soil population is difficult because of the vast numbers that are present. Some heterotrophs gave a browning of the medium, perhaps due to their production of the enzyme tyrosinase. The black discoloration in the MPN tubes could have resulted from sulfate reduction.

Total aerobic plate counts were low, probably due to the presence of the hydrocarbon contaminants which inhibit microorganisms' growth. The first soil sample was contaminated with BTEX's, which are highly volatile; thus, the contaminants were not present in the soil long enough to

allow the microorganisms to begin to degrade them. The extremely volatile nature of BTEX's, combined with the low concentration, did not allow the population of petroleum-decomposing bacteria to reach high enough numbers to be detected. The second soil sample had an extremely high TPH reading of 6000 ppm, which would explain the low counts of microorganisms. Because the concentration of hydrocarbons was not high enough to be toxic to the indigenous flora, the microorganisms able to degrade the contaminant could then proliferate and begin decomposing the contaminant. On the other hand, the introduction of such a high concentration most likely had an initial adverse effect on the microorganisms.

Phenanthrene-degraders: The MPN's using phenanthrene were found to have high counts which can be attributed to organic contaminants introduced in the inoculum. These organic contaminants probably originated from the soil used for the dilutions. These high counts would then be a result of simple heterotrophs proliferating in the medium without phenanthrene degradation. Uninoculated controls were used as an index for the appearance of phenanthrene in the mineral medium. The phenanthrene in positive tubes appeared more dispersed and took on a snowy appearance when the tubes were shaken. There were tubes present which exhibited turbidity, but the appearance of the phenanthrene remained the same as that in the control tubes. Inoculum from these tubes was used as dilutions on phenanthrene overlayer plates to test the ability of the microorganisms growing in the mineral medium with phenanthrene. Although growth occurred on the phenanthrene plates, no halos of clearing were observed. The growth can be explained perhaps by the carbon content in the water or on the glassware used.

Also, carryover of carbon material could have occurred from the inoculum.

The phenanthrene overlayer plates from the second soil sample also exhibited growth without halos. These plates, however, were inoculated with dilutions straight from the soil. The unexplained growth, as observed in the first sample, may also be attributed to carbon contaminants in the water, soil, or on the glassware. Perhaps to help prevent unexplained growth, glassware must be extensively washed and rinsed to insure that it is carbon free. Carbon-free water should be used when making media and buffers to ensure no growth of simple heterotrophs.

Addition of Fertilizer: All freezer bags showed an overall decline in the concentration of hydrocarbon. The freezer bag that received aeration and fertilizer showed the sharpest decline initially, but was equal in hydrocarbon concentration after 30 d. Freezer bags 3 and 4 had the lowest readings after the 30 d extraction. Bag 2 showed an unexpected low reading perhaps due to an error in the extraction process. Freezer bag 2 had the addition of mercury chloride, serving as a bactericidal agent. The initial 13 d extraction was high, but the 30 d extraction was low in hydrocarbon concentration. This suggests that because the microorganisms were inhibited by the mercury chloride, the degradation process was decreased. Bag 1 showed a decline in hydrocarbons even though no additions were made. After the 30 d incubation, bag 1 was lower (.57%) in hydrocarbon concentration than bag 5. From this information, aerating and adding fertilizer served as catalysts in beginning the degradation process. However, the control bag which was

left alone demonstrated equal degradation after the longer period of time (30 d), which may be due to evaporation of the hydrocarbons.

Isolates: The following microorganisms were isolated from phenanthrene overlayer plates: Actinomycete(*Micromonospora*), *Aeromonas*, Coryneforms/*Xanthomonas* (all tentative characterizations). These bacteria were indigenous to the soil and were assumed to be at least partly responsible for the degradation of the contaminants. This assumption was made on the basis of the microorganisms' ability to degrade phenanthrene, the target compound used in the medium. Further study is necessary to determine the enzymes and pathways these microorganisms use to enable them to degrade such hydrocarbons.

CONCLUSION

It was found that the presence of hydrocarbons in soil affected the populations of microorganisms. The overlayer technique used for the phenanthrene plates performed adequately in isolating phenanthrene degraders, even though non-phenanthrene degraders were able to grow on the plates. The ability of isolated microorganisms to degrade phenanthrene was a good indicator that these were responsible for the bioremediation. Aeration and fertilization were shown to increase the rate of bioremediation; and yet, the long-term effects of natural wheathering, as seen in those ecosystems receiving no treatment, resulted in comparable decreases for petroleum hydrocarbons.

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